LPS-mediated effects and spatio-temporal expression of TLR2 and TLR4 in the bovine corpus luteum

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Abstract: When given intravenously (iv), lipopolysaccharide (LPS) transiently suppresses the structure and function of the bovine corpus luteum (CL). This is associated with increased release of prostaglandin (PG) F2 metabolite (PGFM). The underlying regulatory mechanisms of this process remain, however, obscure. Therefore, the aims of this study were: (1) to investigate the expression of the LPS receptor toll-like receptor (TLR) 4 and TLR2 in the bovine CL during early, mid, and late luteal phases; (2) to further dissect the mechanisms of LPS-mediated suppression of luteal function. As revealed by semi-quantitative qPCR and immunohistochemistry, both receptors were detectable throughout the luteal lifespan. Their mRNA levels increased from the early towards the mid-luteal phase; no further changes were observed thereafter. The TLR4 protein seemed more highly represented than TLR2. The cellular localization of TLRs was in blood vessels; weaker signals were observed in luteal cells. Additionally, cows were treated either with LPS (iv, 0.5 µg/kgBW) or with saline on Day 10 after ovulation. Samples were collected 12 h after treatment, and on Day 10 of the respective subsequent (untreated) cycle. The mRNA expression of several possible regulatory factors was investigated, revealing the suppression of PGF2 receptor (PTGFR), steroidogenic acute regulatory protein (STAR) and 3 -hydroxysteroid dehydrogenase (3 HSD), compared with controls and subsequent cycles. The expression of TLR2 and TLR4, interleukin (IL) 1 and IL1 , and of PGF2 and PGE2 synthases (20 HSD/PGFS and mPTGES, respectively) was increased. The results demonstrate the presence of TLR2 and TLR4 in the bovine CL, and implicate their possible involvement in the deleterious effects of LPS on its function.

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Short title: Spatio-temporal expression of luteal TLR2 and TLR4

Abstract

When given intravenously (iv), lipopolysaccharide (LPS) transiently suppresses the structure and function of the bovine corpus luteum (CL). This is associated with increased release of prostaglandin (PG) $F_{2\alpha}$ metabolite (PGFM). The underlying regulatory mechanisms of this process remain, however, obscure. Therefore, the aims of this study were: (1) to investigate the expression of the LPS receptor toll-like receptor (*TLR*) *4* and *TLR2* in the bovine CL during early, mid, and late luteal phases; (2) to further dissect the mechanisms of LPS-mediated suppression of luteal function. As revealed by semi-quantitative qPCR and immunohistochemistry, both receptors were detectable throughout the luteal lifespan. Their mRNA levels increased from the early towards the mid-luteal phase; no further changes were observed thereafter. The TLR4 protein seemed more highly represented than TLR2. The cellular localization of TLRs was in blood vessels; weaker signals were observed in luteal cells. Additionally, cows were treated either with LPS (iv, 0.5 μg/kgBW) or with saline on Day 10 after ovulation. Samples were collected 12 h after treatment, and on Day 10 of the respective subsequent (untreated) cycle. The mRNA expression of several possible regulatory factors was investigated, revealing the suppression of $PGF_{2\alpha}$ receptor (*PTGFR*), steroidogenic acute regulatory protein (*STAR*) and 3β-hydroxysteroid dehydrogenase (*3βHSD*), compared with controls and subsequent cycles. The expression of *TLR2* and *TLR4*, interleukin (*IL*) *1α* and *IL1β*, and of $PGF_{2\alpha}$ and PGE_2 synthases (*20αHSD/PGFS* and *mPTGES*, respectively) was increased. The results demonstrate the presence of TLR2 and TLR4 in the bovine CL, and implicate their possible involvement in the deleterious effects of LPS on its function.

Key words: corpus luteum, toll-like receptor, inflammation, endotoxin, cow

1. Introduction

Inflammatory diseases, especially of the uterus and the mammary gland, reduce fertility in dairy cows (Hansen *et al.* 2004, Sheldon *et al.* 2009a). Infertility or subfertility in cows with uterine inflammation might be exclusively due to local effects in some cases, whereas a systemic inflammatory response is required to decrease fertility in cows with inflammation outside the genital tract, *e.g.*, mastitis (Hansen *et al.* 2004). A systemic inflammatory response due to bovine metritis and mastitis is often associated with the presence of lipopolysaccharide (LPS), the endotoxin of gram-negative bacteria (Bannerman *et al.* 2004, Williams *et al.* 2005). There is increasing evidence that, at least in part, *Escherichia (E.) coli* LPS causes infertility by interfering with ovarian function (Suzuki *et al.* 2001, Herath *et al.* 2007, Lavon *et al.* 2008).

Ovarian dysfunction due to LPS in cows with inflammatory diseases might be due to impairment of the hypothalamic-pituitary axis and interference with ovarian follicular and luteal function (Hansen *et al.* 2004). Thus, cows with uterine infections after parturition were less likely to ovulate, probably because of slower growth of dominant follicles, lower peripheral estradiol (E_2) concentrations, and perturbation of hypothalamic and pituitary function (Sheldon *et al.* 2009b). LPS was found in the follicular fluid of such animals, likely additionally contributing to the delay in ovulation (Sheldon *et al.* 2009b). Similar effects associated with a delayed LH surge and the resulting postponed ovulation were observed after intravenous or intramammary administration of LPS in cows (Lavon *et al.* 2008). Furthermore, intravenous treatment with LPS transiently suppressed progesterone (P_4) secretion by the bovine corpus luteum (CL) and increased plasma concentrations of prostaglandin (PG) $F_{2\alpha}$ metabolites (PGFM) (Herzog *et al.* 2012). Cows with uterine infections after parturition had increased concentrations of LPS, not only in the uterine fluid, but also in plasma and follicular fluid (Mateus *et al.* 2003, Williams *et al.* 2007, Magata *et al.* 2015). Thus, it may be assumed that LPS directly reaches the CL. Indeed, since LPS induced

apoptosis in luteal cell cultures (Grant *et al.* 2007, Mishra & Dhali 2007), the possibility of a direct effect of LPS on the bovine CL should be considered. The direct effects of LPS on target organs depend, however, on the local presence of its specific receptor, toll-like receptor (TLR) 4 (Gerold *et al.* 2007, Kannaki *et al.* 2011), the mRNA expression of which has recently been shown in the bovine mid-cycle CL (Lüttgenau *et al.* 2016).

Whereas TLR2 recognizes bacterial lipids such as lipoteichoic acid and peptidoglycan from gram-positive bacteria, TLR4 in a complex with its co-receptors cluster of differentiation (CD) 14 and myeloid differentiation factor (MyD) 2 binds LPS, leading to signal transduction and activation of the innate immune system (Beutler 2004). However, expression of both *TLR2* and *TLR4* mRNA increased in bovine mammary (Ibeagha-Awemu *et al.* 2008, Ma *et al.* 2011) and endometrial epithelial cells (Fu *et al.* 2013) stimulated with LPS. Furthermore, these studies indicate that TLR4 is present on cells other than leukocytes. In cows, *TLR4* mRNA and protein were detected in endometrial stromal and epithelial cells by RT-PCR and flow cytometry (Herath *et al.* 2006). Activation of TLRs by LPS was observed in endometrial (Fu *et al.* 2013) as well as in mammary epithelial cells (Ibeagha-Awemu *et al.* 2008), thus inducing the downstream signaling cascade that culminates in the secretion of proinflammatory cytokines. In bovine granulosa cells, TLR4 was detected and an inflammatory response to LPS was observed and linked to reduced fertility, due to reduced follicular steroidogenesis (Herath *et al.* 2007). Since murine granulosa cells increased the expression of TLR4 in response to LPS challenge (Shimada *et al.* 2006), granulosa cells seem to have immune capabilities (Herath *et al.* 2007). However, to the best of our knowledge, there are no reports available describing the spatio-temporal expression of TLR2 and TLR4 on the protein level in the bovine CL.

Consequently, the aims of the present study were: (1) to provide evidence for the expression of TLR2 and TLR4 in the bovine CL throughout the luteal phase, to determine their cellular localization; and (2) using samples derived from our previous study (Herzog *et al.* 2012), to

98 further characterize the underlying mechanisms of transient suppression of luteal function
99 after intravenous LPS-treatment by analysing the mRNA expression of cytokines and factors
100 associated with prostaglandin synthesis and steroidogenesis. Expression analyses in the
101 subsequent cycles (after the treated cycles) were aimed at detecting possible carry-over effects
102 of LPS.

2. Materials and Methods

2.1. Study 1: Expression of TLR2 and TLR4 in bovine CL throughout the luteal phase

2.1.1. Corpus luteum collection

Ovaries with CL were harvested from the carcasses of fourteen (n=14) clinically healthy cows (*Bos taurus*), including Red Holstein (n=12), Holstein Friesian (n=1), and Red Holstein x Limousin crossbred (n=1), that were slaughtered at a commercial abattoir. Before slaughter, cows were housed in a tie stall barn, and a modified ovulation synchronization (Ovsynch) protocol was started (at different times) after normal cyclic activity had been ultrasonographically confirmed in each cow. The protocol consisted of 10 µg buserelin (GnRH analog, Receptal®; MSD Animal Health GmbH, Luzern, Switzerland), 15 mg luprostiol ($PGF_{2\alpha}$ analog, Prosolvin®; Virbac AG, Glattbrugg, Switzerland) 7 days (d) later, and finally 10 µg buserelin 60 hours (h) after $PGF_{2\alpha}$ (all treatments were given intramuscularly). Ovulation occurred in all cows within 36 h after the second GnRH treatment. Starting 17 d after ovulation, transrectal B-mode ultrasonography was performed in all cows at 2-d intervals to detect the time of ovulation (Day 1), and subsequently every 2 to 3 d to monitor normal development of the CL. Blood sampling and ultrasonography (B-mode and Power mode) of the CL were performed within 6 h before the cows were slaughtered (at different cycle stages), and ovaries with CL were collected from the carcasses. For each cow, the time points during the luteal phase were randomly selected and allotted to the following groups according to Miyamoto et al. (2000): early (Days 5 to 7; n=4), mid (Days 8 to 12; n=5) or late (Days 13 to 18; n=5) luteal phase.

2.1.2. Collection of blood and analysis of progesterone

Blood samples were collected from the coccygeal blood vessels into evacuated tubes containing EDTA as anticoagulant (Vacuette® 9ml K3EDTA; Greiner bio-one,

Kremsmünster, Austria). Tubes were immediately placed in ice and plasma was separated by centrifugation (3,000 x *g*, 15 min), and frozen at -20 °C until analyses were performed. Concentrations of P_4 were measured using a radioimmunoassay (RIA kit IM1188; Beckman Coulter GmbH, Krefeld, Germany). The range of standard concentrations for this test was 0.03 to 53 ng/mL, intra- and inter-assay coefficients of variation were ≤8.5% and ≤8.7%, respectively, and 50% of relative binding was reached at 1.6 ng/mL.

2.1.3. Ultrasonography

Transrectal ultrasonographic examinations of the uterus and ovaries were performed using a portable ultrasound device (GE LOGIQ e Premium BT11; General Electric Medical System, Solingen, Germany), equipped with a 4.0 to 12.0 MHz linear-array transducer. B-mode and Power Mode ultrasonography and a computer-assisted image analysis software (PixelFlux Version 1.0; Chameleon Software, Leipzig, Germany) were applied to determine luteal tissue area (LTA), luteal blood flow (LBF), and relative LBF (rLBF; LBF divided by LTA). A detailed description of the methodology was provided in previous studies (Lüttgenau *et al.* 2011a, Lüttgenau *et al.* 2011b). In cows showing ovulation of two dominant follicles between Days 0 and 1 (double ovulation), and subsequent development of two CLs, their sum was applied for luteal measurements, as suggested by Bollwein *et al.* (2002).

2.1.4. Preparation of luteal tissue

Immediately after collection, the CL was removed from the ovary, trimmed of connective tissue, incubated for 24 h in RNAlater® (Ambion Biotechnologie GmbH, Wiesbaden, Germany) at 4 °C, and finally stored at -80 °C until analysis. For immunohistochemistry (IHC), tissue samples were fixed for 24 h in 10% neutral phosphate-buffered formalin, washed with phosphate-buffered saline (PBS), dehydrated in a graded ethanol series, and embedded in paraffin-equivalent Histo-Comp® (Vogel Medizintechnik, Giessen, Germany).

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156 *2.1.5. Expression analysis*

157 Luteal mRNA expression was determined for *TLR2*, *TLR4*, steroidogenic acute regulatory
158 protein (*STAR*) and 3β-hydroxysteroid dehydrogenase (*3βHSD*). Accordingly, total RNA
159 from luteal tissue samples was isolated using TRIzol® reagent following the manufacturer's
160 protocol (Invitrogen, Carlsbad, CA, USA) and as described in Kowalewski *et al.* (2013).
161 Semi-quantitative real time (TaqMan) PCR was carried out in an automated fluorometer ABI
162 PRISM® 7500 Sequence Detection System (Applied Biosystems by Thermo Fisher, Foster
163 City, CA, USA) according to a previously described protocol (Kowalewski *et al.* 2006).
164 Samples were run in duplicates with Fast Start Universal Probe Master (ROX)® from Roche
165 Diagnostics (Mannheim, Germany). Autoclaved water instead of RNA and the so called RT
166 minus control were used as negative controls. Integrity of RNA and the assay procedure were
167 tested by amplification of different independent endogenous references (*SDHA*, *GAPDH* and
168 *βACTIN*). The assays were set up to ensure approximately 100% efficiency of the PCR
169 reactions. Relative gene expression was calculated using the comparative CT method (ΔΔCT
170 method) according to the manufacturer's protocols for the ABI PRISM® 7500 Sequence
171 Detection System (Applied Biosystems) and as described previously (Kowalewski *et al.* 2006,
172 Kowalewski *et al.* 2011).
173 Specificity of the selected PCR products was confirmed by sequencing (Microsynth, Galbach,
174 Switzerland). A detailed description of the RT-qPCR method is provided in Kowalewski *et al.*
175 (2006, 2011, 2013). The primers used to amplify specific fragments referring to selected
176 genes purchased either from Microsynth or from Applied Biosystems are presented in
177 Table 1.

178

179 *2.1.6. Immunohistochemistry (IHC)*

Methods of indirect immunoperoxidase IHC were described in detail by Kowalewski *et al.* (2006, 2011, 2013). In brief, luteal cross sections (2-3 µm thick) were cut and mounted on Super-Frost-Plus microscope slides. Antigen retrieval was done using citrate buffer (pH 6.0). Non-specific binding sites were blocked with normal 10% goat serum. The following antibodies were used: affinity purified polyclonal rabbit anti-TLR2 (Biorbyt, Cambridge, UK), and affinity purified polyclonal rabbit anti-TLR4 (Abbiotec, San Diego, CA, USA). The concentration of both primary antibodies was 1:200. Rabbit IgG (Vector Laboratories, Ltd., Burlingame, CA, USA) was used at the same protein concentration as the isotype specific negative control. The secondary antibody was biotinylated goat anti-rabbit IgG (BA-1000) from Vector Laboratories, Ltd. (dilution 1:100). Peroxidase activity was visualized using the DAB substrate kit (Dako Schweiz AG, Baar, Switzerland) and slides were counterstained with hematoxylin.

2.2. Study 2: LPS-induced alterations in the expression of TLR2, TLR4 and other luteal factors

2.2.1. Corpus luteum biopsies

Corpus luteum biopsies from a previous experiment (Herzog *et al.* 2012) were used. In that study, transrectal ultrasonography was performed in each of seven clinically healthy, non-lactating German Holstein cows (*Bos taurus*) at 12, 24, and 36 h after the second GnRH treatment of a modified Ovsynch protocol to detect the time of ovulation (defined as Day 1 of the estrous cycle). On Day 10, cows were treated with 10 mL saline (NaCl 0.9%; iv, during 1-min), and luteal tissue was collected for biopsy 12 h after treatment and additionally on Day 10 of the subsequent (untreated) cycle. Then, the Ovsynch protocol was repeated, and cows were treated with 0.5 µg/kg body weight *E. coli* LPS (O55:B5; Sigma-Aldrich, Taufkirchen, Germany; diluted in 10 mL sterile water; iv, during 1-min) on Day 10 of the

estrous cycle. Again, collection of luteal tissue was performed at 12 h after treatment and on Day 10 of the subsequent (untreated) cycle.

The collection of biopsy samples (approximately 15 x 1 x 1 mm each) from the maximum diameter (including cells from the periphery and the center) of the CL was performed using a semi-automatic, high-speed biopsy needle (TEMNO Evolution™; Fa. Walter, Baruth/Mark, Germany) as described previously (Herzog *et al.* 2012).

This method allowed repeated biopsy sampling from a single CL without impairing its subsequent function (Tsai *et al.* 2001, Atli *et al.* 2012).

2.2.2. Expression analysis

Semi-quantitative real time PCR was applied for investigating the mRNA expression levels of target genes: *TLR2*, *TLR4*, *STAR*, *3βHSD*, cyclooxygenase 2 (*COX2*, *PTGS2*), PGE_2 synthase (*mPTGES*), $PGF_{2\alpha}$ synthase (*20αHSD*/*PGFS*), $PGF_{2\alpha}$ receptor (*PTGFR*, *FP*), fibroblast growth factor 1 (*FGF1*) and -2 (*FGF2*), interleukin *(IL) 1α* and *IL1β*, interleukin 1 receptor (*IL1R1*), tumor necrosis factor α (*TNFα*), TNF receptor 1 (*TNFR1*) and -2 (*TNFR2*). The methodological approach was as presented above for Study 1. The primers and TaqMan probes sequences were purchased from Microsynth (Galbach, Switzerland) and are presented cumulatively in Table 1. The commercially available TaqMan systems for *FGF1*, *FGF2*, *IL1α*, *IL1β*, *IL1R1*, *TNFα*, and *βACTIN* were purchased from Applied Biosystems.

2.3. Statistical analyses

Due to the uneven distribution of data obtained by semi-quantitative real-time PCR, logarithmic transformation was performed to normalize the data and the geometric means (Xg) ± deviation factors (DF) were calculated for the analysis of target gene expression. The effects of observational group on target gene expression were then calculated by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test.

231 Since ultrasonography data were normally distributed, one-way ANOVA was performed for
232 comparisons between early, mid and late luteal phases.
233 All data were analyzed using the statistical analysis system V9.1 (SAS Institute Inc., Cary,
234 NC, USA) and the statistical software GraphPad3 (GraphPad Software Inc., San Diego, CA,
235 USA), respectively. Differences were considered significant at $P \leq 0.05$.

3. Results

3.1. Study 1: Expression of TLR2 and TLR4 in bovine CL throughout the luteal phase

Luteal mRNA expression of *TLR2* and *TLR4* was detectable in all samples investigated at selected time points during the luteal phase, revealing a significant effect of time (ANOVA $P < 0.05$ and $P = 0.02$ for *TLR2* and *TLR4*, respectively). Both receptors showed a similar expression pattern with significantly ($P < 0.05$) increasing mRNA levels from the early to mid-luteal phase; no further changes were observed towards the late luteal phase (Fig. 1A, B). Immunohistochemistry localized the expression of TLR2 and TLR4 predominantly to steroidogenic cells and luteal blood vessels (Fig. 2). In general, especially in luteal cells, TLR4 seemed to be more strongly expressed than TLR2, as indicated by more intense staining of TLR4 which could already be observed during the early luteal phase. During the mid-luteal phase, the signal intensity appeared stronger with both receptors staining intensively, especially in large luteal cells. Within blood vessels, the *tunica intima* and *media* stained strongly for both receptors. In particular, vascular endothelial cells clearly revealed positive signals throughout the luteal phase.

Additionally, the luteal mRNA expression of *STAR* and *3βHSD* was investigated. Their expression was time-dependent (ANOVA $P = 0.002$ and $P = 0.01$ for *STAR* and *3βHSD*, respectively). It increased ($P < 0.01$ and $P < 0.05$, respectively) from the early to mid-luteal phase, and decreased (each $P < 0.05$) thereafter to initial values in the late luteal phase (Supplementary Fig. 1A, B).

Mean LTA was numerically increased ($P > 0.05$) in the mid-luteal phase compared with the early and late luteal phase (Supplementary Table 1). There was no significant difference in absolute and relative LBF as well as plasma P_4 concentrations between the early, mid and late luteal phase.

3.2. Study 2: LPS-induced alterations in the expression of TLR2, TLR4 and other luteal factors

Expression of *TLR2* mRNA was higher ($P < 0.05$) after intravenous LPS-challenge compared with the control cycle (Fig. 3A). However, mRNA abundance of *TLR2* in the estrous cycles that followed the LPS-challenge cycle and the control cycle did not differ from mRNA abundance in the LPS-challenge cycle or from that in the control cycle. Luteal mRNA expression of *TLR4* was increased ($P < 0.05$) after LPS-challenge compared with the control and subsequent cycles after the LPS-challenge and control cycles (Fig. 3B). There was no difference in mRNA abundance of *TLR4* between the control cycle and the subsequent (untreated) cycles. Luteal mRNA expression of the steroidogenic factors *STAR* and *3βHSD* was decreased ($P < 0.001$) after LPS-challenge compared to the control cycle as well as compared to the subsequent cycles after the LPS-challenge and control cycles (Fig. 3C, D). Within prostaglandin-related factors, LPS-challenge significantly increased mRNA abundance for *COX2*, *mPTGES* and *20αHSD/PGFS*, but decreased mRNA for *PTGFR* (Fig. 4A-D). Specifically, *COX2* mRNA expression was higher ($P < 0.01$) in the LPS-challenge cycle compared to the subsequent cycles after the LPS-challenge and control cycles but did not differ significantly between the LPS-challenge cycle and control cycle (Fig. 4A). Luteal expression of *mPTGES* and *20αHSD/PGFS* mRNA was higher ($P < 0.001$) in the LPS-challenge cycle compared to the control cycle and the subsequent cycle after the control cycle (Fig. 4B, C). However, mRNA abundance of *mPTGES* was also higher ($P < 0.001$) in the LPS-challenge cycle compared to the subsequent cycle, whereas mRNA abundance of *20αHSD/PGFS* in the subsequent cycle after the LPS-cycle did not differ significantly from the LPS-cycle or from the control cycle. Luteal mRNA expression of *PTGFR* decreased ($P < 0.001$) after LPS-challenge compared to the control and subsequent cycles (Fig. 4D). Within angiogenic factors, expression of *FGF1* mRNA also decreased ($P < 0.05$) in the LPS-

challenge cycle compared to the control and subsequent cycles, whereas mRNA abundance of *FGF2* increased ($P < 0.05$) after LPS-challenge compared to the other cycles (Fig. 4E, F). Expression levels of *IL1α* and *IL1β* mRNA were increased ($P < 0.001$) after LPS-challenge compared to the control and subsequent cycles (Fig. 5A, B). Luteal mRNA abundance of *IL1R1* was also highest after LPS-challenge but it did not differ from values observed in the control cycle (Fig. 5C). However, *IL1R1* mRNA was more abundant ($P < 0.01$) in the LPS-cycle compared with the untreated cycle that followed the control cycle. Luteal expression of *TNFα* mRNA increased ($P < 0.01$) after LPS-challenge compared to the control and subsequent cycles (Fig. 5D). The mRNA abundance of *TNFR1* did not differ between cycles, whereas mRNA for *TNFR2* was increased ($P < 0.001$) in the LPS-cycle compared with the other cycles (Fig. 5E, F).

4. Discussion

The results of the present study establish, for the first time, expression patterns of TLR2 and TLR4 in the bovine CL during the luteal phase. On the mRNA level, the expression of *TLR2* and *TLR4* has recently been shown in the mid-cycle CL of isolated perfused bovine ovaries *in vitro* (Lüttgenau *et al.* 2016), which could be confirmed in the present *ex vivo* study. The cellular localization of both receptors was in steroidogenic cells and luteal vessels, indicating an immune capability of those cells directed towards components of gram-positive as well as gram-negative bacteria. Therefore, effects of LPS on the bovine CL do not seem to be restricted to TLR-bearing immune cells that are normally present in luteal tissue as reported in a review by Walusimbi & Pate (2013). The localization of TLR4 in luteal cells was consistent with previous reports that established the presence of TLR4 in granulosa (Herath *et al.* 2007, Bromfield & Sheldon 2011, Price & Sheldon 2013) and theca cells (Magata *et al.* 2014) from ovarian follicles, which are the progenitors of the large and small luteal cells, respectively (Alila & Hansel 1984).

The mRNA levels of *TLR2* and *TLR4* increased from the early towards the mid-luteal phase and remained unchanged afterwards, *i.e.*, during the late luteal phase. The expression patterns of *STAR* and *3βHSD* mRNA were determined in order to validate the allocation of CLs to their respective experimental groups (early, mid or late luteal phase). Increased expression of *STAR* and *3βHSD* between the early and mid-luteal phase, and decreased expression between the mid and late luteal phase, were consistent with changes in steroidogenic capacity of the CL throughout its lifespan (Herzog *et al.* 2010). Determination of plasma P_4 concentrations, luteal size and blood flow further confirmed that only tissue derived from functional CLs was used.

Escherichia coli LPS given intravenously increased luteal mRNA expression of *TLR2* and *TLR4*. Because TLR4 is the receptor for the gram-negative endotoxin LPS (Poltorak *et al.*

1998), the increase in *TLR4* mRNA might indicate an autoamplification pathway that supports increased binding of LPS. Binding and activation of TLR4 initiates the production of pro-inflammatory cytokines and leads to the recruitment of leukocytes (Sheldon & Bromfield 2011). However, in the recent study (Lüttgenau *et al.* 2016) that used isolated perfused ovaries, luteal mRNA expression of *TLR4* did not differ between LPS-treated and control ovaries during the first 3 h after treatment. Apart from the differences in the general approach of the recent and the present study (*in vitro* vs. *in vivo*), the different outcome in luteal expression of several factors that were investigated in both studies might be due to the different sampling time. Whereas biopsies in the present study were performed at 12 h after treatment, luteal tissue from isolated perfused ovaries was collected during the first 3 h after challenge because viability of the ovary could not be guaranteed for a longer time. In both studies, LPS increased TLR2, which typically recognizes bacterial lipids from gram-positive bacteria (Takeda & Akira 2005). Increased expression of *TLR2* mRNA was also observed in mammary glands challenged with LPS (Ibeagha-Awemu *et al.* 2008) as well as *E. coli* (Yang *et al.* 2008). Although the reason for increased *TLR2* mRNA after LPS treatment is not known, studies in mice (Matsumura *et al.* 2000) and humans (Davanian *et al.* 2012) suggest that LPS-induced TNFα increases *TLR2* mRNA and protein expression. In accordance with this, in the present study, *TNFα* mRNA was also increased after LPS-challenge.

The decrease in luteal mRNA expression of steroidogenic factors *STAR* and *3βHSD* after LPS-challenge was consistent with the transient decrease in plasma P_4 concentrations reported by Herzog *et al.* (2012). Both STAR and 3βHSD catalyze key steps of steroidogenesis (Couët *et al.* 1990, Stocco & Clark 1996) and were inhibited by $PGF_{2\alpha}$ (Stocco *et al.* 2007). The increase in mRNA expression of factors related to prostaglandin synthesis (*COX2*, *mPTGES* and *20αHSD/PGFS*) was in accordance with increased plasma concentrations of PGFM and PGE_2 after LPS-treatment (Herzog *et al.* 2012). It is known that pulsatile release of $PGF_{2\alpha}$ from the uterus or administration of $PGF_{2\alpha}$ increase luteal $PGF_{2\alpha}$ synthesis (Stocco *et al.*

2007, Shirasuna *et al.* 2010). The increase in luteal expression of both luteolytic $PGF_{2\alpha}$ and luteotropic PGE_2 might explain the absence of complete premature luteolysis reported previously (Herzog *et al.* 2012). In contrast, luteal mRNA expression of *PTGFR* was decreased after LPS-challenge, possibly due to increased $PGF_{2\alpha}$ concentrations, because *PTGFR* mRNA was also reduced by $PGF_{2\alpha}$ administration (Shirasuna *et al.* 2010).

Luteal mRNA expression of *FGF1* decreased whereas that of *FGF2* increased after LPS-challenge. Fibroblast growth factors are potent mitogens for endothelial cells and other cell types including luteal cells, and are therefore strong luteotropic factors within the CL (Yamashita *et al.* 2008, Shirasuna *et al.* 2010). The mechanisms that decrease FGF1 but increase FGF2 remain unknown; however, different expression levels of these luteotropic factors might have contributed to the incomplete luteolysis observed by Herzog *et al.* (2012).

In the present study, LPS increased luteal mRNA expression of the proinflammatory cytokines *IL1α*, *IL1β* and *TNFα*. Increased concentrations of TNFα (in milk and plasma) were also observed after LPS-induced mastitis (Hoeben *et al.* 2000). Interleukins and TNFα mediate the inflammatory response at both the local and systemic levels by promoting neutrophil transendothelial migration to the site of infection and by inducing fever and the acute-phase response (Bannerman *et al.* 2004). Furthermore, IL1α, IL1β and TNFα are potent stimulators of luteal prostaglandins including $PGF_{2\alpha}$ and PGE_2 (Nishimura *et al.* 2004, Sakumoto & Okuda 2004). Consistent with this, luteal mRNA expression of *20αHSD/PGFS* and *mPTGES* was increased after LPS-challenge in the present study. Both TNFα receptors, TNFR1 and TNFR2, are expressed in the bovine CL (Korzekwa *et al.* 2008) but only the mRNA abundance of *TNFR2* was increased after LPS-challenge.

In the estrous cycles that followed the LPS-challenge and control cycles, luteal expression of none of the investigated parameters differed from that observed in the control cycles, indicating the absence of any carry-over effect of LPS on the CL in the subsequent cycle.

In conclusion, the expression of TLR2 and TLR4 was predominantly localized to luteal cells and blood vessels and increased during the mid and late luteal phase. It seems possible that luteal TLR2 and TLR4 are involved in the immune response of luteal tissue to an intravenous application of *E. coli* LPS *in vivo*, which is associated with the production of proinflammatory cytokines and reduced ovarian steroidogenesis in cows.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legends

Fig. 1. Relative levels (Xg ± DF) of luteal mRNA expression of *TLR2* (A) and *TLR4* (B) during the early (Days 5 to 7; n=4), mid (Days 8 to 12; n=5) and late (Days 13 to 18; n=5) luteal phases (Day 1 = ovulation). Parametric ANOVA ($P \leq 0.05$) was applied, followed by the Tukey-Kramer Multiple Comparisons test; (**) indicates $P < 0.05$.

Fig. 2. Immunohistochemical localization of TLR2 and TLR4. Representative photographs are shown at Day 6 (early luteal phase), Day 11 (mid-luteal phase), and at Day 17 (late luteal phase). Open arrows = luteal cells, solid arrows = luteal vessels.

Fig. 3. Relative levels (Xg ± DF) of luteal mRNA expression of *TLR2* (A), *TLR4* (B), *STAR* (C), and *3βHSD* (D) on Day 10 of the estrous cycle (Day 1 = ovulation) at 12 h after intravenous treatment with LPS (*E. coli* O55:B5; 0.5 µg/kgBW) or saline (control), and on Day 10 of the subsequent (2^{nd}) cycle after the LPS-challenge and control cycles. Parametric ANOVA ($P \leq 0.03$) was applied, followed by the Tukey-Kramer Multiple Comparisons test; ^{a,b} Values with different superscripts differ ($P < 0.05$) between the indicated cycles.

Fig. 4. Relative levels (Xg ± DF) in luteal mRNA expression of *COX2* (A), *mPTGES* (B), *20αHSD/PGFS* (C), *PTGFR* (D), *FGF1* (E) and *FGF2* (F) on Day 10 of the estrous cycle (Day 1 = ovulation) at 12 h after intravenous treatment with LPS (*E. coli* O55:B5; 0.5 µg/kgBW) or saline (control), and on Day 10 of the subsequent (2^{nd}) cycle after the LPS-challenge and control cycles. Parametric ANOVA ($P \leq 0.02$) was applied, followed by the Tukey-Kramer Multiple Comparisons test; ^{a,b} Values with different superscripts differ ($P < 0.05$) between the indicated cycles.

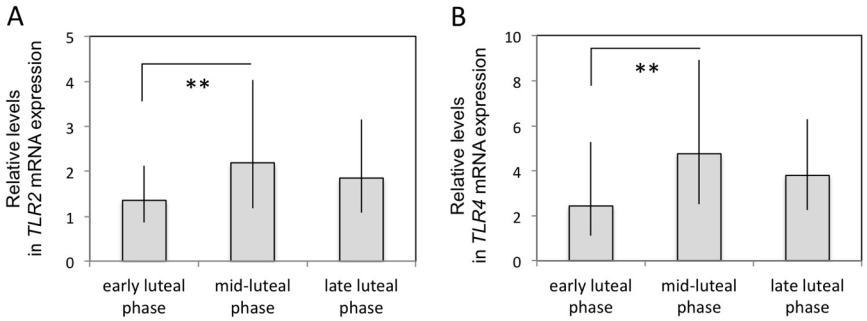
Fig. 5. Relative levels (Xg ± DF) of luteal mRNA expression of *IL1α* (A), *IL1β* (B), *IL1R1* (C), TNF*α* (D), *TNFR1* (E), and *TNFR2* (F) on Day 10 of the estrous cycle (Day 1 = ovulation) at 12 h after intravenous treatment with LPS (*E. coli* O55:B5; 0.5 µg/kgBW) or saline (control), and on Day 10 of the subsequent (2^{nd}) cycle after the LPS-challenge and control cycles. Parametric ANOVA ($P \leq 0.006$; except for *TNFR1* with $P > 0.05$) was applied, followed by the Tukey-Kramer Multiple Comparisons test; ^{a,b} Values with different superscripts differ ($P < 0.01$) between the indicated cycles.

Supplementary Fig. 1. Relative levels (Xg ± DF) of luteal mRNA expression of *STAR* (A) and *3βHSD* (B) during the early (Days 5 to 7; n=4), mid (Days 8 to 12; n=5) and late (Days 13 to 18; n=5) luteal phases (Day 1 = ovulation). Parametric ANOVA ($P \leq 0.05$) was applied, followed by the Tukey-Kramer Multiple Comparisons test; (**) indicates $P < 0.05$, (***) indicates $P < 0.01$.

Table 1. Accession numbers and sequences of PCR primers for assayed genes from bovine corpus luteum cells, and length of PCR products

Primer	Accession Numbers	Primer Sequences	Product Length
TLR2	NM_174197	Forward: 5`-TCCACGGACTGTGGTACATGA-3` Reverse: 5`-ACACGAAGGCGTCGTAGCA-3` TaqMan probe: 5`-CCAGGAAGGCTCCCCGCAGG-3`	102
TLR4	NM_174198	Forward: 5`-AAGACTGGGTGCGGAATGAAC-3` Reverse: 5`-CCTTACGGCTTTTGTGGAAACC-3` TaqMan probe: 5`-TGGCCATCGCCGCCAATATCATC-3`	144
STAR	NM_174189	Forward: 5`-AAGTCCCTCAAGGACCAAACTC-3` Reverse: 5`-TGCGAGAGGACCTGGTTGAT-3` TaqMan probe: 5`-ACCTCAAGGGATGGCTGCCGAAGA-3`	90
3βHSD	NM_174343	Forward: 5`-CACACCGCCTCTGTCATTGA-3` Reverse: 5`-GTACGCTGGCCTGGACACA-3` TaqMan probe: 5`-TGCTGTCCCGCGAGACCATCA-3`	112
COX2 (PTGS2)	NM_174445	Forward: 5`-GCACAAATCTGATGTTTGCATTC-3` Reverse: 5`-GGTCCTCGTTCAAAATCTGTCT-3` TaqMan probe: 5`-TTGCCCAGCACTTCACCCATCAATT-3`	76
mPTGES (PGES)	NM_174443	Forward: 5`-CAAGTGAGGCTGCGGAAGA-3` Reverse: AGGCAGCGTTCCACATCTG-3` TaqMan probe: 5`-TTTGCCAACCCCGAGGACGCTC-3`	101
20αHSD/ PGFS (AKR1B5)	NM_001012519	Forward: 5`-ACCTGGACCTCTACCTCATCCA-3` Reverse: 5`-TCCTCATCCAATGGGAAGAAGT-3` TaqMan probe: 5`-CCCACAGGCTTCAAGCCTGGGA-3`	73
PTGFR (FP)	NM_181025	Forward: 5`-GCCAACTGGAAGAAGACCTTTC-`3 Reverse: 5`-CTGGTATGCCTTCATGAGGATAGC-3` TaqMan probe: 5`-CAGTGGGAATCTTATCGAACAGCCTGGC-3`	101
FGF1	NM_174055.2	Applied Biosystems, prod. nr.: Bt03212662_m1	67
FGF2	NM_174056.3	Applied Biosystems, prod. nr.: Bt03259205_m1	100
IL1α	NM_174092	Applied Biosystems, prod. nr.: Bt03212739_m1	94
IL1β	NM_174093	Applied Biosystems, prod. nr.: Bt03212745_m1	129
IL1R1	NM_001206735	Applied Biosystems, prod. nr.: Bt04300521_m1	94

TNFα	NM_173966.3	Applied Biosystems, prod. nr.: Bt03259154_m1	84
TNFR1	NM_174674.2	Forward: 5`-GTTATGTCCAACCCGACCTTCA-3` Reverse: 5`-GGCAAAGCCCGAAGACAAT-3` TaqMan probe: 5`-AAGACTCTCAGGACCCAGG-3`	92
TNFR2	NM_001040490.2	Forward: 5`-GTCACCGCATGCTTTAGCTGTA-3` Reverse: 5`-TGGCTTGCAGGTGCAGATG-3` TaqMan probe: 5`-AACTCAAGCCTGCACAAC-3`	99
SDHA	NM_174178	Forward: 5`-ATGGAAGGTCTCTGCGCTAT-3` Reverse: 5`-ATGGACCCGTTCTTCTATGC-3` TaqMan probe: 5`-ACAGAGCGATCACACCGCGG-3`	119
GAPDH	NM_001034034	Forward: 5`-GCGATACTCACTCTTCTACCTTCGA-3` Reverse: 5`-TCGTACCAGGAAATGAGCTTGAC-3` TaqMan probe: 5`-CTGGCATTGCCCTCAACGACCACTT-3`	82
βACTIN	NM_173979.3	Applied Biosystems, prod. nr.: Bt03279175_g1	144



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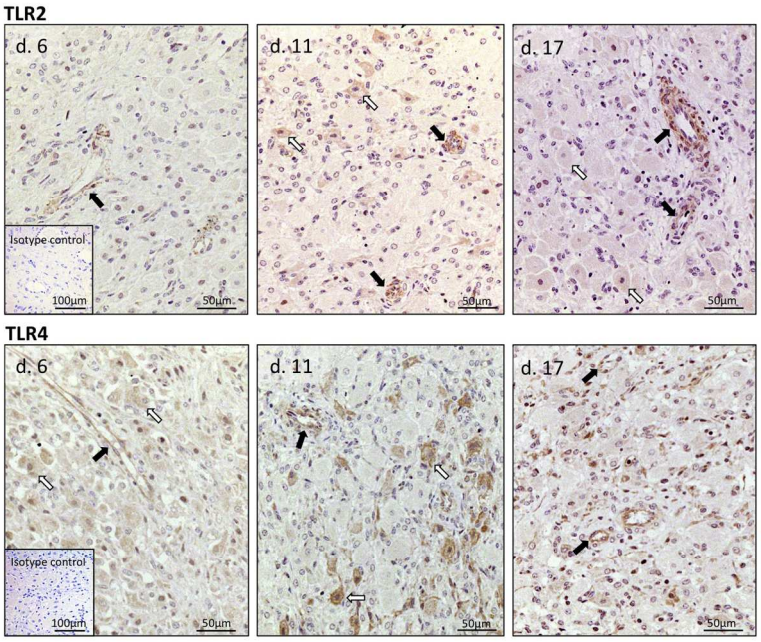

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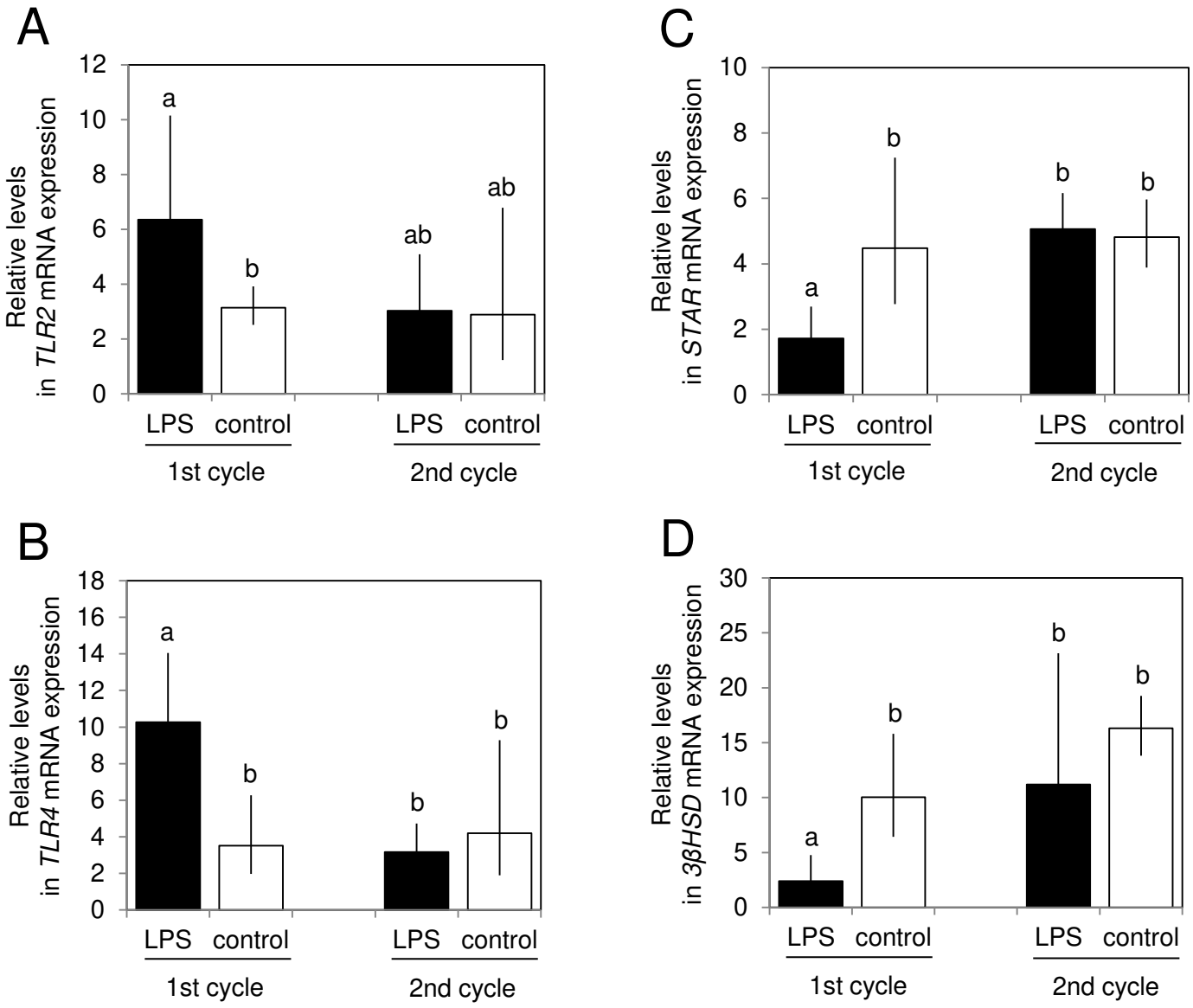
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Relative levels in TLR4 mRNA expression
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1st cycle
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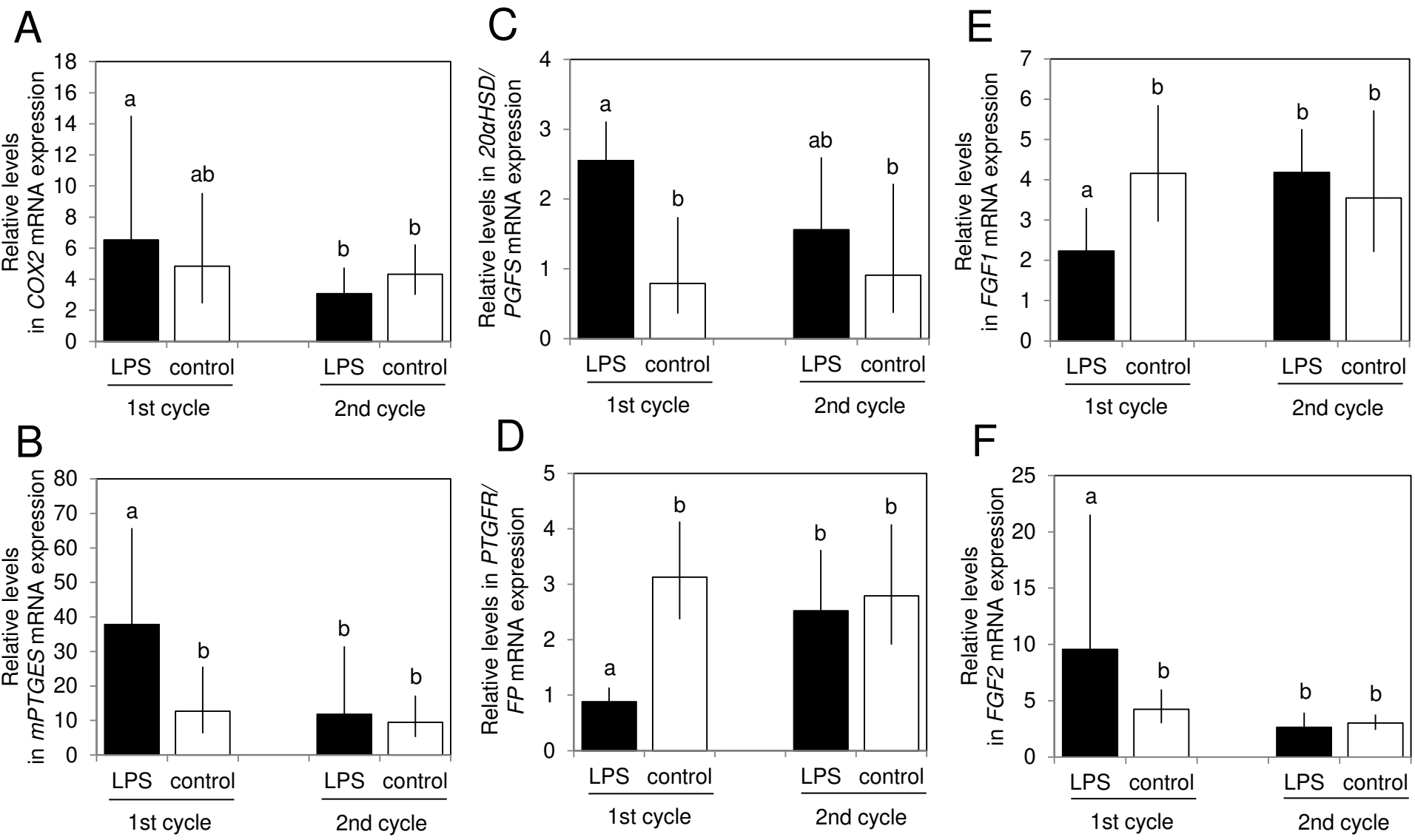
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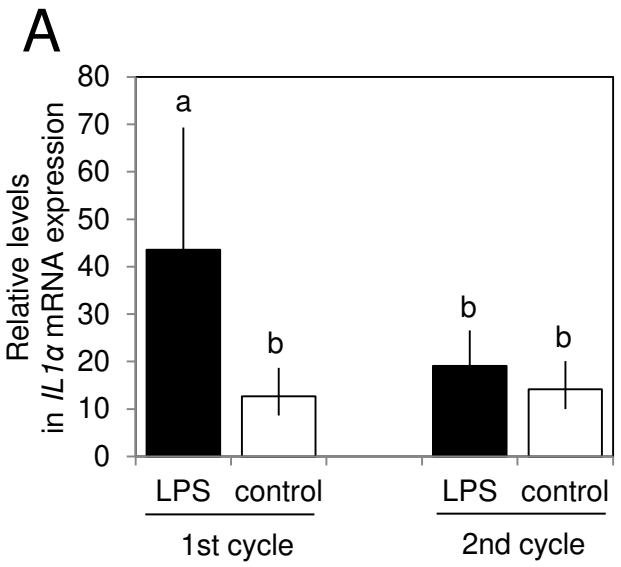
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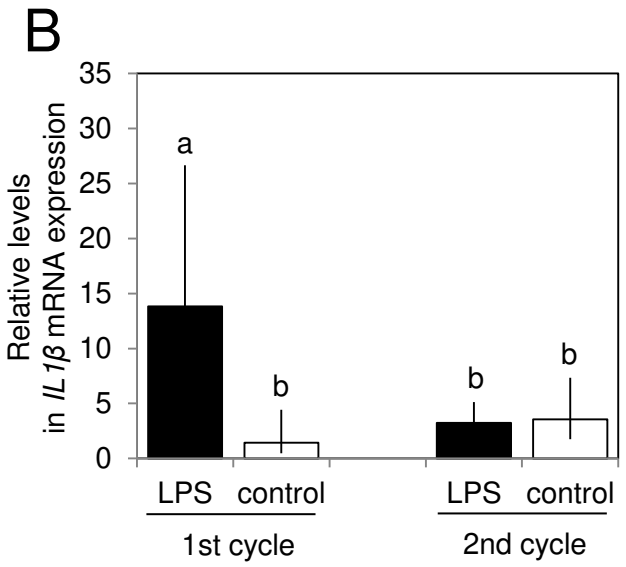
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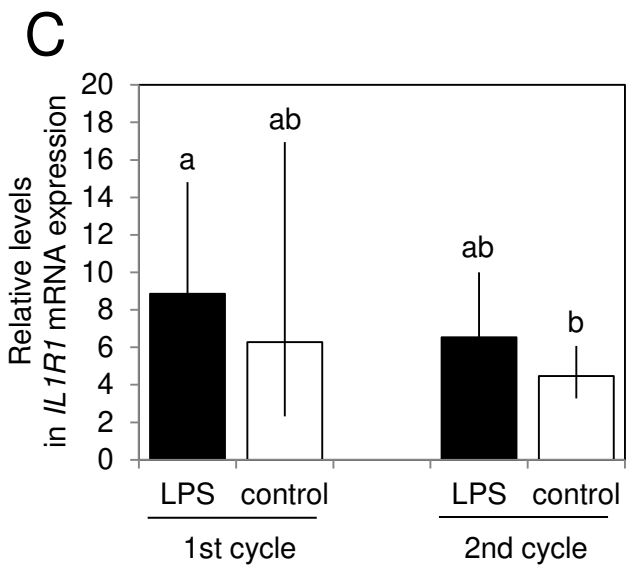
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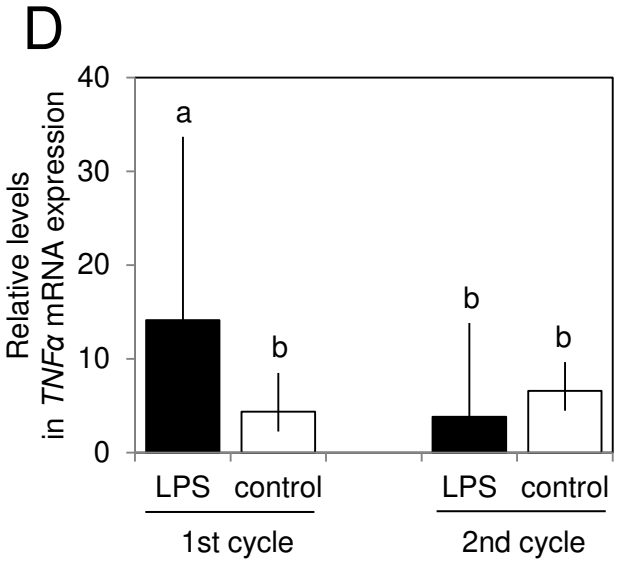
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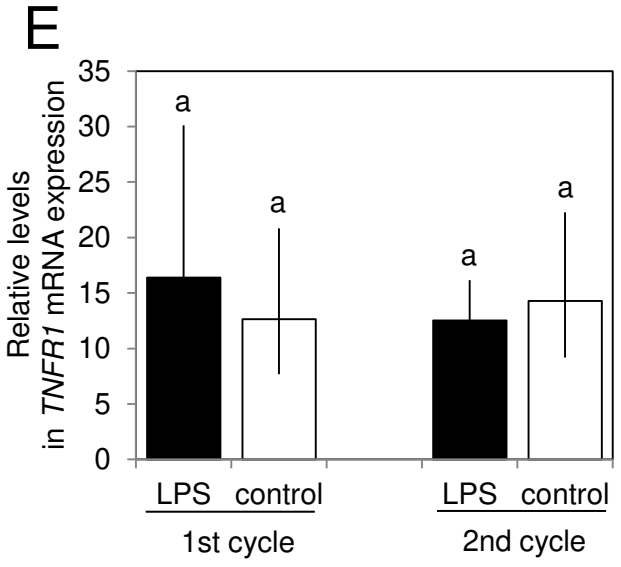
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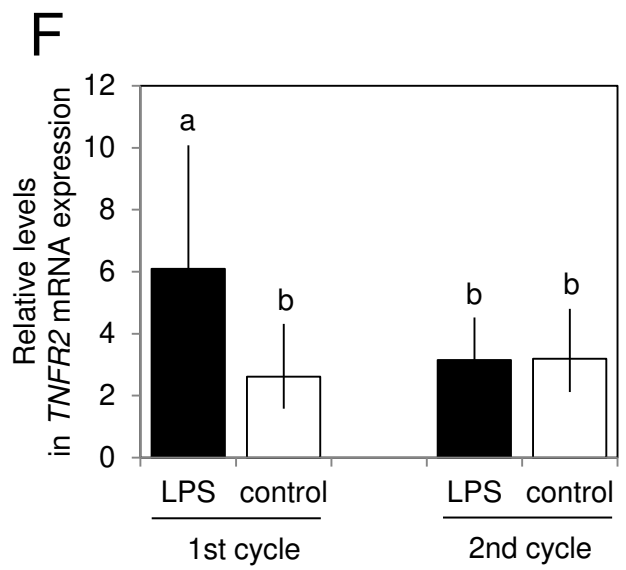
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